

07-19-07 JC03 Filed PCT/PTO 11.7 JUL 2001 PCT



FORM 1390 (Modified) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER
TRANSMITTAL LETTER TO THE UNITED STATES		03528.0133.PCUS00
DESIGNATED/ELECTED OFFICE (DO/EO/US)		U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR
CONCERNING A FILING UNDER 35 U.S.C. 371		Not Yet Assigned 09/889686
INTERNATIONAL APPLICATION NO	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/DE00/03119	05 September 2000	23 November 1999

TITLE OF INVENTION
METHOD FOR THE CONTROLLED POST-HARVEST PRODUCTION OF PROTEINS IN HOST ORGANISMS

APPLICANT(S) FOR DO/EO/US
Klaus DURINGand Lorenz BULOW

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

- 1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
- 2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
- 2. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)). The submission must include itens (5), (6), (9) and (24) indicated below.
- 4. ☒ The US has been elected by the expiration of 19 months from the priority date (Article 31).
- 5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
 - a. ☒ is attached hereto (required only if not communicated by the International Bureau).
 - b. ☐ has been communicated by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
- 6. ☒ An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
 - a. ☒ is attached hereto.
 - b. ☐ has been previously submitted under 35 U.S.C. 154(d)(4).
- 7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
 - a. ☐ are attached hereto (required only if not communicated by the International Bureau).
 - b. ☐ have been communicated by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☒ have not been made and will not be made.
- 8. ☐ An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
- 9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
- 10. ☐ An English language translation of the annexes of the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).
- 11. ☐ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
- 12. ☐ A copy of the International Search Report (PCT/ISA/210).

- Items 13 to 20 below concern document(s) or information included:**
- 13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
 - 14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3 31 is included.
 - 15. ☒ A **FIRST** preliminary amendment.
 - 16. ☐ A **SECOND** or **SUBSEQUENT** preliminary amendment.
 - 17. ☐ A substitute specification.
 - 18. ☐ A change of power of attorney and/or address letter.
 - 19. ☒ A computer-readable form of the sequence listing in accordance with PCT Rule 13ter.2 and 35 U.S.C. 1.821 - 1.825.
 - 20. ☐ A second copy of the published international application under 35 U.S.C. 154(d)(4).
 - 21. ☐ A second copy of the English language translation of the international application under 35 U.S.C. 154(d)(4).
 - 22. ☒ Certificate of Mailing by Express Mail
 - 23. ☒ Other items or information:

Statement Regarding Sequence Submission; Paper form of Sequence Listing; Clean Version of Amended Paragraphs; Clean Version of Pending Claims; Return receipt postcard

24. The following fees are submitted.:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

- ☒ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO \$1000.00
- ☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO \$860.00
- ☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$710.00
- ☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$690.00
- ☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) \$100.00

ENTER APPROPRIATE BASIC FEE AMOUNT =**CALCULATIONS PTO USE ONLY**

\$1,000.00

\$0.00

Surcharge of \$130.00 for furnishing the oath or declaration later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE
Total claims	23 - 20 =	3	x \$18.00
Independent claims	1 - 3 =	0	x \$80.00

\$54.00

\$0.00

Multiple Dependent Claims (check if applicable).

☒

\$270.00

TOTAL OF ABOVE CALCULATIONS =

\$1,324.00

☒ Applicant claims small entity status. (See 37 CFR 1.27). The fees indicated above are reduced by 1/2.

\$662.00

SUBTOTAL =

\$662.00

Processing fee of \$130.00 for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)).

+

\$0.00

TOTAL NATIONAL FEE =

\$662.00

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable).

☐

\$0.00

TOTAL FEES ENCLOSED =

\$662.00

Amount to be:
refunded

\$

charged

\$

- a. ☒ A check in the amount of \$662.00 to cover the above fees is enclosed.
- b. ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed.
- c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 08-3038. A duplicate copy of this sheet is enclosed.
- d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Albert P. Halluin
HOWREY SIMON ARNOLD & WHITE, LLP
301 Ravenswood Avenue
Box 34
Menlo Park, CA 94025
(650) 463-8109

SIGNATURE

Albert P. Halluin, Viola T. Kung

NAME

25,227, 41,131

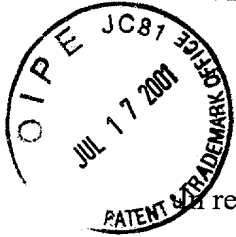
REGISTRATION NUMBER

July 17, 2001

DATE

EXPRESS MAIL NO. EL 615209145US

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE



re application of:

Düring, *et al.*

Application Serial No.: To be Assigned

Filed: Herewith

For: **METHOD FOR THE CONTROLLED
POST-HARVEST PRODUCTION OF
PROTEINS IN HOST ORGANISMS**

Group Art Unit: To be Assigned

Examiner: To be Assigned

Attorney's Docket No:
03528.0133.PCUS00

PRELIMINARY AMENDMENT

Box DO/EO/US
Commissioner for Patents
Washington, D.C. 20231

Sir:

Applicants submit herewith a Preliminary Amendment. The Examiner is respectfully requested to enter the amendments prior to considering the application.

THE AMENDMENT

In the Specification

Page 1, line 3, before "The present invention" insert --This application is a National Stage of International Application PCT/DE00/03119, filed September 5, 2000; which claims the priority of DE 199 56 272.5, filed November 23, 1999.--

Page 14, line 23, following "CATGTCAACACATAAGGAAGAAGAGGTAGAAAG" insert --(SEQ ID NO: 1)--.

Page 14, line 25, following "CATGCCATGGATCGATGACGGGGTTGGCGAGTGTG" insert --(SEQ ID NO: 2)--.

Page 14, line 31, following "CATGCCATGGCATG" insert --(SEQ ID NO: 3)--.

Page 14, line 33, following "GCTCTAGAGC" insert --(SEQ ID NO: 4)--.

Page 16, line 38, following: "CATGCCATGCCACAATTTGATATATTATGTAAAAC"
insert --(SEQ ID NO: 5)--.

Page 16, line 39, following: "GCTCTAGATCAGACTGTGGCAGGGAAACCCTC"
insert --(SEQ ID NO: 6)--.

In the Claims

1. (Amended) A method of obtaining a desired protein from a transgenic host organism, wherein the expression of the gene coding for this protein is not made until the host organism has been harvested, [characterized in that] wherein
 - (a) the transgenic host organism contains the gene coding for the desired protein such that it is only expressed in the presence of a chemical inductor; and
 - (b) contacting with the inductor takes place via the phase surrounding the host organism after the host organism has been harvested.
4. (Amended) The method according to claim 2, wherein step (b) comprises [modification of] modifying the gas phase surrounding the host organism, [atomization of] atomizing a solution [(suspension)] or a suspension of an inductor or flooding with a volatile inductor.
5. (Amended) The method according to claim 3, wherein step (b) comprises [an infection] infecting with a virus suspension.
6. (Amended) The method according to any one of claims 1 to 5, wherein the gene coding for the desired protein is functionally linked with an inducible promoter.
7. (Amended) The method according to [any of claims 2, 4 and 6] claim 4, wherein [the modification of] modifying the gas phase is [an deoxidation] deoxidizing the gas phase and the promoter is a promoter inactive under aerobic conditions.

9. (Amended) The method according to [any of claims 2, 4 and] claim 6, wherein in step (b) contacting with the [chemical] inductor takes place via atomization of the inductor RH5992.
10. (Amended) The method according to any one of claims 1 to 3, wherein the expression of the gene coding for the desired protein is induced by compensating the functional inhibition of the transcription and/or translation.
11. (Amended) A method according to claim 10, wherein the gene coding for the desired protein is functionally linked with a promoter, so that between the promoter and the gene a nucleic acid is inserted [which is characterized in] such that
- (a) it prevents the transcription and/or translation of the gene; and
 - (b) it can be excised after the induction, which results in the expression of the gene.

Cancel Claim 15.

16. (Amended) The method according to [any of claims 1 to 15] claim 1, wherein the transgenic organism is a useful plant.

REMARKS

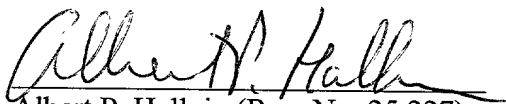
The Amendment

The above amendments correct the improper format of multiple dependent claims. The amendments also change the European style claims to proper method and composition claims.

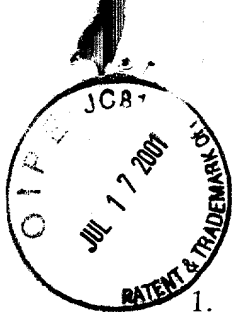
No new matter is added in any of the amendments. The Examiner is respectfully requested to enter all the amendments.

Respectfully submitted,

Date: July 17, 2001


Albert P. Halluin (Reg. No. 25,227)
Viola T. Kung (Reg. No. 41,131)

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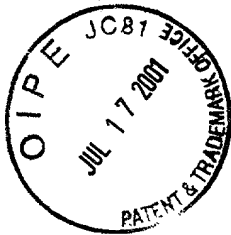


CLEAN VERSION OF ALL PENDING CLAIMS

1. A method of obtaining a desired protein from a transgenic host organism, wherein the expression of the gene coding for this protein is not made until the host organism has been harvested, wherein
 - (a) the transgenic host organism contains the gene coding for the desired protein such that it is only expressed in the presence of a chemical inductor; and
 - (b) contacting with the inductor takes place via the phase surrounding the host organism after the host organism has been harvested.
2. The method according to claim 1, wherein the phase is a gas phase.
3. The method according to claim 1, wherein the phase is a liquid phase.
4. The method according to claim 2, wherein step (b) comprises modifying the gas phase surrounding the host organism, atomizing a solution or a suspension of an inductor or flooding with a volatile inductor.
5. The method according to claim 3, wherein step (b) comprises infecting with a virus suspension.
6. The method according to any of one of claims 1 to 5, wherein the gene coding for the desired protein is functionally linked with an inducible promoter.
7. The method according to claim 4, wherein modifying the gas phase is deoxidizing the gas phase and the promoter is a promoter inactive under aerobic conditions.
8. The method according to claim 7, wherein the promoter is the GapC4 promoter.

9. The method according to claim 6, wherein in step (b) contacting with the inductor takes place via atomization of the inductor RH5992.
10. The method according to any one of claims 1 to 3, wherein the expression of the gene coding for the desired protein is induced by compensating the functional inhibition of the transcription and/or translation.
11. A method according to claim 10, wherein the gene coding for the desired protein is functionally linked with a promoter, so that between the promoter and the gene a nucleic acid is inserted such that
 - (a) it prevents the transcription and/or translation of the gene; and
 - (b) it can be excised after the induction, which results in the expression of the gene.
12. The method according to claim 11, wherein the nucleic acid is a nucleic acid which can be excised by an inducible recombinase.
13. The method according to claim 12, wherein the excisable nucleic acid and the recombinase are constituents of the recombinase-LBD system.
14. The method according to any of claims 1 to 3, wherein the gene coding for the desired protein is expressed by compensating the effect of the transcriptional, post-transcriptional, translational or post-translation repressor.
16. The method according to claim 1, wherein the transgenic organism is a useful plant.
17. The method according to claim 16, wherein the useful plant is wheat, barley, corn, sugar beet, sugarcane, potato, a brassicaceae, a leguminous plant or tobacco.

18. A host organism according to claim 1, which contains the gene coding for the desired protein such that it is only expressed in the presence of a chemical inductor.



CLEAN VERSION OF AMENDED PARAGRAPHS

Page 1, line 3:

This application is a National Stage of International Application PCT/DE00/03119, filed September 5, 2000; which claims the priority of DE 199 56 272.5, filed November 23, 1999. The present invention relates to a method of obtaining a desired protein from a transgenic host organism, the gene coding for this protein being not expressed until the host organism has been harvested and the method being characterized in that said gene is only expressed in the presence of a chemical inductor supplied after the harvest of the host organism thereto via the surrounding phase, in particular gas or liquid phase.

Page 14, line 23:

HincII-pGapC4 primer: CATGTCAACACATAAGGAAGAAGAGGTAGAAAG
(SEQ ID NO: 1) pGapC4-NcoI primer:
CATGCCATGGATCGATGACGGGGTTGGCGAGTGTG (SEQ ID NO: 2)

Page 14, line 28:

The cDNA described by Artsaenko *et al.*, Molecular Breeding 4 (1998), 313-319, which codes for an scFv antibody localized in the endoplasmic reticulum, was modified by means of a linker ligation (at the 5' end with CATGCCATGGCATG, (SEQ ID NO: 3) [5'-phosphorylated oligonucleotide]; at the 3' end with GCTCTAGAGC (SEQ ID NO: 4) [5'-phosphorylated oligonucleotide] such that it had an NcoI restriction site at the 5' end and an XbaI restriction site at the 3' end. The CaMV 35S promoter was removed from plasmid pRT100 (Töpfer *et al.* Nucleic Acids Research 15 (1987), 5890) by means of restriction digestion using HincII and XbaI. The two above described nucleic acid fragments which code for the GapC4 promoter and the scFv antibody, were inserted instead. After partial cleavage

with HindIII, the expression cassette was isolated and inserted in the binary vector pSR 8-30 (Düring *et al.*, Plant Journal 3, (1993), 587, 598; Porsch *et al.*, Plant Molecular Biology 37 (1998), 581-585). The expression vector pSR 8-30/Gap-scFv(ox) was obtained.

Page 16, line 38:

NcoI-PLP-LBD primer: CATGCCATGCCACAATTTGATATATTATGTAAAAC
(SEQ ID NO: 5)

FLP-LBD-XbaI primer: GCTCTAGATCAGACTGTGGCAGGGAAACCCTC
(SEQ ID NO: 6)

The expression cassette from pRT 100/FLP was inserted as partially digested PstI fragment between the two FRT recombination sites. As a result, the plasmid pRT 100/rec-scFv(ox) was obtained.



IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Düring, *et. al*

Application Serial No. To Be Assigned

Filed: herewith

For: **METHODS FOR THE CONTROLLED
POST-HARVEST PRODUCTION OF
PROTEINS IN HOST ORGANISMS**

Attorney's Docket No:
035280133PCUS00

STATEMENT REGARDING SEQUENCE SUBMISSION

BOX/DO/EO/US
Commissioner for Patents
Washington, D.C. 20231

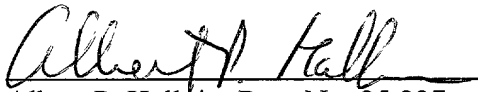
Sir:

In accordance with 37 C.F.R. § 1.821(f), the paper copy of the Sequence Listing and the computer readable copy of the Sequence Listing submitted herewith in the above-mentioned application are the same.

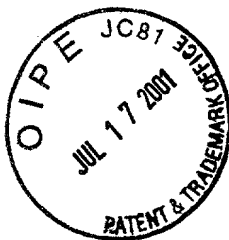
In accordance with 37 C.F.R. § 1.821(g), this submission includes no new matter.

Respectfully submitted,

Date: July 17, 2001


Albert P. Halluin, Reg. No. 25,227
Viola T. Kung, Reg No. 41,131

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09/889686

JC17 Rec'd PCT/PTO 17 JUL 2001

SEQUENCE LISTING

<110> DÜRING, Klaus
BÜLOW, Lorenz

<120> METHOD FOR THE CONTROLLED POST-HARVEST
PRODUCTION OF PROTEINS IN HOST ORGANISMS

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32

(12) NACH DEM VERTRAG ÜBER DIE INTERNATIONALE ZUSAMMENARBEIT AUF DEM GEBIET DES
PATENTWESENS (PCT) VERÖFFENTLICHTE INTERNATIONALE ANMELDUNG

(19) Weltorganisation für geistiges Eigentum
Internationales Büro



(43) Internationales Veröffentlichungsdatum
31. Mai 2001 (31.05.2001)

PCT

(10) Internationale Veröffentlichungsnummer
WO 01/38508 A2

(51) Internationale Patentklassifikation⁷: C12N 15/00

(74) Anwalt: HUBER, Bernard; Patentanwälte Huber & Schüssler, Truderinger Strasse 246, D-81825 München (DE).

(21) Internationales Aktenzeichen: PCT/DE00/03119

(22) Internationales Anmeldedatum:
5. September 2000 (05.09.2000)

(81) Bestimmungsstaaten (*national*): AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW.

(25) Einreichungssprache: Deutsch

(26) Veröffentlichungssprache: Deutsch

(84) Bestimmungsstaaten (*regional*): ARIPO-Patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), eurasisches Patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), europäisches Patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI-Patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

(30) Angaben zur Priorität:
199 56 272.5 23. November 1999 (23.11.1999) DE

(71) Anmelder (*für alle Bestimmungsstaaten mit Ausnahme von US*): MPB COLOGNE GMBH, MOLECULAR PLANT & PROTEIN BIOTECHNOLOGY [DE/DE]; Neurather Ring 1, D-51063 Köln (DE).

Veröffentlicht:

— Ohne internationalen Recherchenbericht und erneut zu veröffentlichen nach Erhalt des Berichts.

(72) Erfinder; und

(75) Erfinder/Anmelder (*nur für US*): DÜRING, Klaus [DE/DE]; Vorgebirgsweg 33, D-50226 Frechen-Königsdorf (DE). BÜLOW, Lorenz [DE/DE]; Werder 2, D-38100 Braunschweig (DE).

Zur Erklärung der Zweibuchstaben-Codes, und der anderen Abkürzungen wird auf die Erklärungen ("Guidance Notes on Codes and Abbreviations") am Anfang jeder regulären Ausgabe der PCT-Gazette verwiesen.

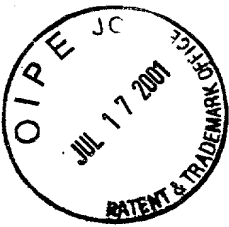
(54) Title: METHOD FOR CARRYING OUT THE CONTROLLED POST-HARVEST PRODUCTION OF PROTEINS IN HOST ORGANISMS

(54) Bezeichnung: VERFAHREN ZUR GESTEUERTEN NACH-ERNT-PRODUKTION VON PROTEINEN IN WIRTSORGANISMEN

(57) Abstract: The invention relates to a method for extracting a desired protein from a transgenic host organism, whereby the expression of the gene that codes for this protein is firstly carried out after the host organism has been harvested. The inventive method is characterized in that: (a) the transgenic host organism contains the gene that codes for the desired protein in such a way that the expression thereof is firstly carried out in the presence of a chemical inductor, and; (b) the gene is brought into contact with the inductor via the phase surrounding the host organism after the host organism has been harvested. The invention also relates to a host organism that is suited for carrying out said method.

(57) Zusammenfassung: Beschrieben wird ein Verfahren zur Gewinnung eines gewünschten Proteins aus einem transgenen Wirtsorganismus, wobei die Expression des für dieses Protein codierende Gens erst nach der Ernte des Wirtsorganismus erfolgt und das Verfahren dadurch gekennzeichnet ist, daß (a) der transgene Wirtsorganismus das für das gewünschte Protein codierende Gen derart enthält, daß dessen Expression erst in Anwesenheit eines chemischen Induktors erfolgt und (b) das Inkontaktbringen mit dem Induktor nach der Ernte des Wirtsorganismus über die den Wirtsorganismus umgebende Phase erfolgt. Ferner betrifft die Erfindung einen zur Durchführung dieser Verfahrens geeigneten Wirtsorganismus.

WO 01/38508 A2



09/889686
JC17 Rec'd PCT/PTO 17 JUL 2001

**Method for the Controlled Post-Harvest Production of
Proteins in Host Organisms**

The present invention relates to a method of obtaining a desired protein from a transgenic host organism, the gene coding for this protein being not expressed until the host organism has been harvested and the method being characterized in that said gene is only expressed in the presence of a chemical inductor supplied after the harvest of the host organism thereto via the surrounding phase, in particular gas or liquid phase.

Proteins occurring in nature are often available in only very small amounts, nevertheless their characteristics for use as active substances and materials are highly interesting. Since they can often also be obtained in recombinant host systems, e.g. bacteria, such as *Escherichia coli*, *Bacillus subtilis*, etc., in non-efficient manner regarding the economical conditions and sufficient amounts, a commercial application cannot be realized in such cases. In order to be able to produce more and more complex proteins which are hard to produce, or cannot be produced at all, in low organisms, an increasing number of cells of higher organisms with their inherent complex protein biosynthesis machinery is additionally required as host cells. Transgenic animals, plants, fungi, moss, algi, etc., have offered themselves as new recombinant host in this case for some years now. Since the number of well characterized available proteins from molecular research increases constantly, this technology is gaining significance as regards their application.

However, the expression of foreign proteins may have negative effects on the physiological constitution of the host organism, and e.g. impair its growth or even prevent it. In addition it is conceivable that e.g. if transgenic plants expressing medically active proteins are cultivated, these

09/889686

represent a danger potential for organisms in the environment while the plants grow. Therefore, the post-harvest production of foreign proteins in transgenic host organisms, in particular those which are cultivated by means of agriculture or horticulture, is of great significance as a technological building block towards an economical and environmentally compatible extraction of proteins. Other (bio)chemical substances can also be prepared in transgenic host organisms by the expression of enzymes involved in the biosynthesis route thereof. However, this also involves the risk of negative effects on the host organisms, e.g. growth inhibitions, or as regards the biological safety. Since the production of (bio)chemical substances in host organisms is also based on the expression of proteins, in particular enzymes, the same technological standards apply here as well.

Only post-harvest production systems have been described thus far which are based on the wounding of plant material, e.g. in the case of tobacco plants, by comminuting their leaves. As a result, the desired protein shall not yet be produced while the tobacco plant is grown in the field but only after the harvest prior to the recovery step. However, this is difficult to realize, since a wounding-inducible promoter is induced in transgenic plants in agricultural or horticultural cultivation, e.g. in tobacco, already in the case of damage caused by feeding, wind or hail, impact, use of machines, etc. The uniform distribution, which is hard to achieve, of the induction stimulus over the entire plant substance to be induced is another disadvantage of the former method.

Thus, the present invention is based on the technical problem of providing a post-harvest production system for a desired protein which does not have the drawbacks of the method described in the prior art, i.e. ensures above all that gene expression takes place reliably only after the harvest, comminution of the plant tissue is not necessary and the inductor is contacted efficiently and uniformly with the cells of the corresponding transgenic host organism.

This technical problem is solved by the method defined in the claims according to the invention.

In the method according to the invention the expression of a foreign gene is induced in a host organism by a chemical inductor administered via the phase surrounding the host organism. This phase may be a gas or liquid phase, wherein in the former phase the chemical induction may take place by changing the composition of the gas phase, preferably an anaerobic induction; an atomization of solutions (suspensions) of solid or liquid inducing (bio)chemicals or a use of volatile substances and their uniform distribution in the reaction chamber. Furthermore, the chemical induction may take place in the liquid phase via solutions of solid or liquid inducing (bio)chemicals. High-quality proteins or other (bio)chemical substances can be produced by the method according to the invention on a large scale and thus for new (bio)medical and technological fields of application.

Thus, the present invention comprises a method of obtaining a desired protein from a transgenic host organism, the gene coding for this protein being expressed only after the harvest of the host organism and the method being characterized in that

- (a) the transgenic host organism contains the gene coding for the desired protein such that it is only expressed in the presence of a chemical inductor; and
- (b) contacting with the chemical inductor takes place after the harvest of the host organism via the phase surrounding the host organism, in particular gas or liquid phase.

A person skilled in the art is familiar with methods of constructing the nucleic acid constructs required for carrying out the method according to the invention, and these methods are also described in common standard works (cf. e.g. Sambrook et al., 1989, Molecular Cloning, A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY). The nucleic acid construct is

preferably available in that it is inserted on a vector, the vector being preferably a plasmid, a cosmid, a virus, a bacteriophage, or another vector common in genetic engineering. These vectors may have further functional units which stabilize the vectors in the host organism, such as a bacterial replication origin or the 2-mikron-DNA for stabilization in *Saccharomyces cerevisiae*. "Left border" and "right border" sequences of agrobacterial T-DNA may also be contained so as to enable stable integration into the genotype of plants. A termination sequence may also be present which serves for properly terminating the transcription and adding a Poly-A sequence to the transcript. Such elements are described in the literature (cf. Gielen et al., EMBO J. 8 (1989), 23-29) and can be exchanged as desired.

For preparing the insertion of a foreign gene in higher plants a large number of cloning vectors are available which contain a replication signal for *E. coli* and a marker gene for the selection of transformed bacterial cells. Examples of such vectors are pBR322, pUC series, M13mp series pACYC184, etc. The foreign gene may be inserted in the vector at a suitable restriction site. The resulting plasmid is used for the transformation of *E. coli* cells. Transformed *E. coli* cells are cultured in a suitable medium, then harvested and lysed so as to obtain the plasmid. In general restriction analyses, gel electrophoreses and further biochemical and molecular-biological methods are used as an analysis method for characterizing the obtained plasmid DNA. Following every manipulation, the plasmid DNA may be cleaved and extracted DNA fragments may be linked with other DNA sequences. Each plasmid DNA sequence may be cloned in the same or other plasmids.

A plurality of techniques are available for the insertion of DNA, e.g. in a vegetable host cell. These techniques comprise the transformation of plant cells with T-DNA using *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes* as the transformation agent, the fusion of protoplasts, the

injection, the electroporation of DNA, the insertion of DNA by means of biolistic methods and further possibilities.

For the injection and electroporation of DNA in plant cells no special demands are made on the plasmids used. It is possible to use simple plasmids, e.g. pUC derivatives. However, if whole plants are regenerated from such transformed cells, a selectable marker should be present. Depending on the method of inserting desired genes in the plant cell, further DNA sequences may be necessary. For example, if the Ti or Ri plasmid is used for the transformation of the plant cell, at least the right border, but often the right and left borders, of the Ti and Ri plasmid T-DNA have to be connected as a flanking region with the genes to be inserted.

If agrobacteria are used for the transformation, it is favorable to clone the DNA to be inserted in special plasmids, in particular in an intermediary or in a binary vector. The intermediary vectors may be integrated due to sequences which are homologous to sequences in the T-DNA, by homologous recombination in the Ti or Ri plasmid of the agrobacteria. This plasmid additionally contains the vir region necessary for the transfer of the T-DNA. Intermediary vectors cannot replicate in agrobacteria. The intermediary vector can, by means of the helper plasmid, be transferred to *Agrobacterium tumefaciens*. Binary vectors may replicate in both *E. coli* and agrobacteria. They contain a selection marker gene and a linker or polylinker which are surrounded by the right and left T-DNA boundary region.

They can be transformed directly into the agrobacteria. The agrobacterium serving as a host cell shall contain a plasmid which carries a vir region. The vir region is necessary for the transfer of the T-DNA into the plant cell. Additional T-DNA may be present. The agrobacterium transformed in this way is used for transforming plant cells.

For transferring the DNA into the plant cell it is possible

to co-cultivate vegetable explants usefully with *Agrobacterium tumefaciens* or *Agrobacterium rhizogenes*. Then, whole plants can be regenerated again from the infected plant material, e.g. leaf portions, stem segments, roots, protoplasts or suspension-cultivated plant cells in a suitable medium which may contain antibiotics or biocides for the selection of transformed cells. The resulting plants may then be tested for the presence of the introduced DNA. Other possibilities are known for introducing foreign DNA using the biolistic method or by protoplast fusion.

Alternative systems for transforming monocotyl plants are the transformation by means of the biolistic approach, the electrically or chemically induced DNA uptake in protoplasts, the electroporation of partially permeabilized cells, the macroinjection of DNA into inflorescence, the microinjection of DNA into microspores and pro-embryos, the DNA uptake by germinating pollen and the DNA uptake into embryos by swelling (for review: Potrykus, *Physiol. Plant* (1990), 269-273). While the transformation of dicotyl plants via Ti-plasmid vector systems by means of *Agrobacterium tumefaciens* is established, more recent studies point out that monocotyl plants are also accessible to transformation by means of vectors based on agrobacterium.

The transgenic host organisms useful for the method according to the invention may, in principle, be plants of any plant species, i.e. both monocotyl and dicotyl plants. Useful plants are preferred, in particular plants, such as wheat, barley, rice, corn, sugar beets, sugarcane, potatoes, brassicaceae, leguminous plants or tobacco. The plant portions desired for the expression of the desired protein or the treatment with the chemical inductors relate, in principle, to any plant portion, in any case to replication material and harvest products of these plants, e.g. fruit, seeds, nodules or bulbs, rootstocks, seedlings, cuttings, etc.

Furthermore, any protein, in particular a diagnostic protein,

a therapeutic protein and/or a material protein, may be produced by the method according to the invention. The protein may originate from any individual, in particular humans or animals. The protein may also be present in wild-type or modified form. It may be a fusion protein or a protein fragment.

In a preferred embodiment, contacting with the chemical inductor is carried out in step (b) of the method according to the invention via the gas phase surrounding the host organism. This is done preferably by modifying the gas phase surrounding the host organism, i.e. the gas phase serves as a carrier for the induction stimulus, by atomizing an inductor solution, or by flooding using a volatile inductor. The modification of the gas phase in the host organism results preferably in an induction, e.g. of the promoter concerned. For physical reasons alone resulting from the utilization of the diffusion effect, a homogenous distribution of the induction stimulus is achieved after a certain time already. The period for reaching this homogenous distribution is considerably shortened by actively circulating the gas phase in the reaction chamber. Uniform and rapid penetration is thus achieved in particular in closely abutting cell substances as in plant leaves, algae or moss tissue. In particular in the case of compact tissues, e.g. potato tubers, the rapid tissue penetration according to the invention is of great advantage.

The person skilled in the art is familiar with suitable gaseous inductors as well as with conditions for an exchange of the gas phase as efficient as possible. Reference is made to the Anaerocult system (Merck, Darmstadt, Germany) which produces an anaerobic environment, in which oxygen is bound and CO₂ is released. In this system, the GapC4 promoter from corn is induced anaerobically by the CO₂ atmosphere (Bülow, L. et al., Molecular Plant-Microbe Interactions (1999), 182-188). The same effect is also achieved by introducing industrial nitrogen. Another example is the induction of "pathogenesis related protein" promoters, such as L-

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phenylalanine ammonium lyase promoters, chalcone synthase or "hydroxyproline rich glycoprotein" promoters by ethylene (Ecker, J.R. and Davis, R.W., Proc. Natl. Acad. Sci USA 84 (1987), 5202-5206).

The person skilled in the art is also familiar with soluble inductors suitable for atomization and with conditions for an atomization as efficient as possible. Reference is made to a chimeric transcription-induction system which is induced by the soluble inductor dexamethasone (Plant J. 11 (1997) 605-612; Kunkel et al., Nature Biotechnol. 17 (1999), 916-918). The Incw1 promoter of corn is activated by the addition of sucrose of D-glucose (Chen, W.H. et al., Proc. Natl. Acad. Sci. U.S.A. 96 (1999), 10512-10517). Many "pathogenesis related protein" promoters are activated by salicylic acid (Gaffney et al., Nature 261 (1993), 754-756). A volatile inductor is methyl salicylate which is converted in the uptaking plant into salicylate which as described above has an inducing effect (Shulaev, V. et al., Nature 385 (1997), 718-721). The atomization of solutions, e.g. of promoter-inducing (bio)chemicals, offer the advantage of a technically simple and uniform distribution of the inducing substance around the tissue to be induced and into the tissue. A rapid and uniform distribution is preferably supported by an active circulation of the gas phase. In addition, a simple and efficient balance of resulting concentration differences is thus achieved, so that controlled process conditions can be guaranteed. In a particularly preferred embodiment, the agrochemical RH5992 (tebufenozide, Rohm & Haas, Croyden, U.K.) is used for the atomization, RH5992 functioning via a chimeric transcription activator protein as an inductor for the promoter to be induced (Gatz and Lenk, Trends in Plant Science 3 (1998), 352-358). The promoter is switched on specifically by RH5992 and is inactive without the presence of this compound. In this case, the inductor mist uniformly approaches the tissue to be induced by a continuous air distribution, for example. The active induction of the promoter is achieved by diffusion from the tissue surface into the cells.

Volatile inductors suitable for flooding and promoters inducible by them are also known to the person skilled in the art. In particular, methyl salicylate has to be mentioned which is converted in the uptaking plant into salicylate which, as described above, has an inducing effect (Shulaev, V. et al., *supra*). Another example of a volatile inductor is ethanol which induces the *alcA* promoter from *Aspergillus nidulans* in transgenic tobacco (Caddick, M.X., et al., *Nature Biotechnology* 16 (1998), 177-180). For active flooding one proceeds by flooding the liquid or solid (volatile) inductor with a suitable gaseous carrier medium for the purpose of conversion into the volatile gaseous phase and an active circulation of the gas phase is preferably carried out to obtain a uniform distribution of the inductor.

In a preferred embodiment, contacting with the chemical inductor takes place in step (b) of the method according to the invention via the liquid phase surrounding the host organism. This is done preferably via solutions of solid or liquid inducing (bio)chemicals. Reference is made to the above statements on the atomization of soluble inductors. It may be favorable to supply the chemical inductor via virus suspensions, e.g. the tobacco mosaic virus (TMV) in the case of tobacco, to the host organism, i.e. infect it therewith.

In a particularly preferred embodiment of the method according to the invention the gene coding for the desired protein is linked with an inducible promoter. Suitable promoters and the inhibitors in consideration for this (gaseous, liquid or solid, volatile compounds) are known to the person skilled in the art. They include e.g. the above described systems *GapC4* promoter / CO₂ (Bülow et al., *supra*), the *Adh1* promoter from corn / anaerobiosis (Ellis et al., *EMBO J.* 6 (1987), 11-16), the L-phenylalanine ammonium lyase promoters, chalcone synthase promoters or "hydroxyproline rich glycoprotein" promoters / ethylene (Ecker, J.R. and Davis, *supra*), the chimeric transcription induction system / dexamethasone (Plant J., *supra*), the *Incw1* promoter from corn / sucrose or D-glucose (Chen, W.H. et al., *supra*), many

"pathogenesis related protein" promoters / salicyclic acid or methyl salicylate (Gaffney et al. *supra*; Shulaev et al., *supra*), the *alcaA* promoter from *Aspergillus nidulans* in transgenic tobacco / ethanol (Caddick, M.X. et al., *supra*).

The promoter is preferably a promoter inactive under aerobic conditions, such as the GapC4 promoter of the *Adh1* promoter, and induction takes place via a modification of the gas phase such that deoxidation is concerned. In this case, the aerobically inducible GapC4 promoter (DE 195 47 272) is particularly preferred, e.g. in combination with harvested transgenic plant tissue, e.g. transgenic potato tubers. Most vegetable promoters are switched off under anaerobic conditions while the GapC4 promoter is switched off under aerobic conditions and is switched on by simple deoxidation (Bülow et al., *supra*). This is achieved e.g. by introducing nitrogen or carbon dioxide into the reaction or storage space. Thus, a fully anaerobic environment is obtained within few hours even in an intact potato tuber so as to achieve promoter induction and foreign protein expression.

In another preferred embodiment of the method according to the invention, the expression of the gene coding for the desired protein is induced by compensating the functional inhibition of the transcription and/or translation. An example of a transcriptional inhibition is the HRT protein (repressor) from barley, which binds to the "gibberellinphytohormone response element" of the α -amylase promoter from barley (Raventos, D. et al., J. Biol. Chem. 273 (1998), 23313-23320). If this repressor is expressed under the control of the CaMV 35S promoter under aerobic conditions in a transgenic plant, it binds to the target sequence which is inserted between the gene coding for the desired protein and its promoter. If anaerobic conditions are produced, the 35S promoter is switched off and the repressor neosynthesis is stopped and hence the above gene is activated by the degradation of the existing repressor.

An example of the translational inhibition is the repression

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of the translation of the ferritin transcript in wheat germ extracts by the expression of a 90 kDa repressor from rat liver which binds to the 5'-untranslated region of the ferritin mRNA (Brown, P.H., J. Biol. Chem. 264 (1989), 13383-13386). The repressor of the ferritin mRNA is inserted before the cDNA sequence coding for the desired protein and the repressor is expressed in the transgenic plant.

The activity of the repressor may also be controlled directly by an inductor or inhibitor. For example, regulated transcriptional activation of the PS-IAA4/5 and PS-IAA6 promoters takes place in *Pisum sativum* by the addition of indoleacetic acid or cycloheximide, so that the transcript is not produced until these activators are present (Koshiba, T. et al., J. Mol. Biol. 253 (1995), 396-413). The translational repression of the tet operator is directly compensated in transgenic arabidopsis plants by e.g. adding tetracycline (Ulmasov, B. et al., Plant Mol. Biol. 35 (1997), 417-424).

In a particularly preferred embodiment, the method according to the invention is characterized in that the gene coding for the desired protein is functionally linked with a promoter such that a nucleic acid is inserted between the promoter and the gene, which is characterized in that (a) it prevents the transcription and/or translation of the gene; and (b) can be excised by means of induction, which results in the expression of the gene. Thus, the transcription or translation of the desired gene is prevented in the non-induced state and thus the foreign protein encoded by this gene does not form either. In an even more preferred embodiment of the method according to the invention, the inserted nucleic acid can be excised by a repressed and inducible recombinase. The gene coding for the recombinase may in this case be inserted separately in the host plant or may be localized itself on the inserted nucleic acid. A locally specific recombination and thus an excision of this nucleic acid sequence takes place only after activating the recombinase by a (bio)chemical physical or genetic inductor, and as a result the desired gene is localized directly

downstream of the promoter, which initiates the transcription and translation and thus the foreign protein biosynthesis. As regards the inductors activating the recombinase reference is made to the above statements on the promoter induction systems. In particular it is mentioned that the recombinase gene may be located on the virus, e.g. TMV, and is not activated until the host organism has been infected.

The recombinase LBD system (WO 95/00555) is most preferred for the method according to the invention. It is inserted between the promoter and the foreign gene to be expressed as follows:

5'-promoter -- R -- recombinase LBD -- R -- foreign gene to be expressed -- terminator -3'

(R = recombination site for recombinase LBD).

The recombinase is inactivated by fusion with the LBD domain. Having harvested the plant material, it is incubated with the inductor, e.g. estradiol, activating the foreign protein production and binding to the LBD domain. As a result, the recombinase is activated and the cDNA fragment coding for recombinase is hence excised at both recombination sites and thus the gene to be expressed is localized directly downstream of the promoter. As a result, the transcription and translation of the gene to be expressed is thus enabled and the foreign protein is produced under controlled conditions.

Another alternative preferred embodiment of the method according to the invention is characterized in that the expression of the gene coding for the desired protein is made by compensating the effect of a transcriptional, post-transcriptional, translational or post-translational repressor, the separation of the repressor from the bound nucleic acid or protein sequence being induced by an external stimulus. Reference is made to the above statements on the compensation of the functional inhibition of transcription and/or translation.

Finally the present invention relates to a method in which various of the above described induction mechanisms are combined, e.g. as a two-component system. As a result, an even more restrictive regulation of the foreign gene expression, e.g. in transgenic plants, and thus an even more increased degree of biological safety can be achieved, at the same time it is possible to stop potential negative effects on the physiology of the plant. For example, a promoter inducible by a gaseous substance, e.g. the anaerobically inducible GapC4 promoter from corn, or a system based on a repressor can be combined with another system, e.g. a system based on recombination, so that the transcription of the respective foreign gene, based on the inducible promoter, is enabled only after combining promoter and gene by controlled recombination. For example, the above described recombinase LBD system (WO 95/00555) can be used as a recombination system, which consists of a 5' recombination site for the recombinase of the cDNA coding for the recombinase LBD protein and a 3' recombination site for the recombinase. In this two-component system, the promoter is inactive under aerobic conditions. The recombinase is also inactivated by the fusion with the LBD domain. Thus there is double repression. The foreign gene is therefore not expressed under agricultural and horticultural conditions, even if one of the two repression systems is not fully blocked by an environmental stimulus. This negative effect would then be absorbed by the second (repression) system). After the harvest of the plant material, the LBD domain-binding inductor is added for the foreign protein production and moreover anaerobic conditions are established in the reaction chamber. This serves for activating the recombinase, on the one hand, and hence the cDNA fragment coding for recombinase is excised at the two recombination sites and thus the gene to be expressed is localized directly at the anaerobic promoter. Then, the transcription and translation of the gene to be expressed are enabled by producing anaerobic conditions and the foreign protein is produced under controlled conditions.

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Another subject matter of the present invention relates to host organisms which contain the gene coding for the desired protein such that it is expressed only in the presence of a chemical inductor. The host organisms are in particular host organisms showing an above described transgenity. Corresponding reference is made to the above statements.

The following examples explain the invention.

Example 1: Anaerobic post-harvest production of scFv in transgenic potatoes

The anaerobically inducible GapC4 promoter (DE 195 47 272) was modified by means of a PCR reaction such that it contained at the 5' end a HincII restriction site and a NcoI restriction site. The following primer pair was used for the PCR reaction:

HincII-pGapC4 primer: CATGTCAACACATAAGGAAGAAGAGGTAGAAAG

pGapC4-NcoI primer:

CATGCCATGGATCGATGACGGGGTTGGCGAGTGTG

The cDNA described by Artsaenko *et al.*, Molecular Breeding 4 (1998), 313-319, which codes for an scFv antibody localized in the endoplasmic reticulum, was modified by means of a linker ligation (at the 5' end with CATGCCATGGCATG, [5'-phosphorylated oligonucleotide]; at the 3' end with GCTCTAGAGC [5'-phosphorylated oligonucleotide] such that it had an NcoI restriction site at the 5' end and an XbaI restriction site at the 3' end. The CaMV 35S promoter was removed from plasmid pRT100 (Töpfer *et al.* Nucleic Acids Research 15 (1987), 5890) by means of restriction digestion using HincII and XbaI. The two above described nucleic acid fragments which code for the GapC4 promoter and the scFv antibody, were inserted instead. After partial cleavage with HindIII, the expression cassette was isolated and inserted in the binary vector pSR 8-30 (Düring *et al.*, Plant Journal 3, (1993), 587, 598; Porsch *et al.*, Plant Molecular Biology 37 (1998), 581-585). The expression vector pSR 8-30/Gap-scFv(ox)

was obtained.

It was used for transforming *E. coli* SM10 (Koncz and Schell, Molecular and General Genetics 204 (1986), 383-396). Transformants were mixed with agrobacterium GV 3101 (Koncz and Schell, *supra*) and incubated overnight at 28°C (Koncz et al., Proc. Natl. Acad. Sci U.S.A. 84 (1987), 131-135). Selection on carbenicillin was carried out, the bla gene necessary for this being present in the above expression vectors. Selection clones of *Agrobacterium tumefaciens* were applied onto cut off leaves, scratched several times at the middle rib, of a potato plant cv. Désirée, and the leaves were incubated in the dark for 2 days at 20°C. Thereafter, the agrobacteria were washed off and plant growth substances were added to the potato leaves, so that preferably shoots regenerated. Furthermore, non-transformed cells were killed in the potato leaves by the addition of kanamycin (100 mg/ml) to the plant medium. Growing shoots were cut off and grew roots in the medium without plant growth substances but with kanamycin. The potato plants were further cultivated as usual.

For detecting the expression of the scFv antibody, cut-off leaf material or intact or cut-off bulb material was induced by means of the "Anaerocult" system (Merck, Darmstadt, Germany) as described by Bülow et al. (1999), *supra*. After 40 hours, the leaf material was removed and mortared. The detection of the expressed scFv antibody was made via the resulting c-myc Tag using the monoclonal antibody 9E10-IgG (Cambridge Research Chemicals, Northwich, Cheshire, U.K.) or protein L (Clontech, Palo Alto, CA, U.S.A.) in a Western blot or ELISA. For this purpose, the whole protein of the potato material was isolated and used in the corresponding detection methods.

Transgenic potato plants determined to be expression-positive were cultivated in a greenhouse (in a pot or in a ground bed) or in the field under common horticultural or agricultural conditions. The tubers were harvested as usual and stored.

For the post-harvest production of the scFv antibody, the tubers were placed in a reaction container made of steel (or plastics) which had a gas supply valve at its bottom and a gas discharge valve at its top. The air in the container was displaced rapidly by the industrial nitrogen (or carbon dioxide). A constant composition of the gas phase in the reaction container was adjusted in a slow gas stream (1 m³ gas supply per hour per m² base area). After 40 hours, the tubers were taken out of the reaction container, homogenized, the solids content was centrifuged off and the aqueous supernatant was supplied for chromatographic purification of the scFv antibody. By a comparison of samples before and after the deoxidation it could be shown that no scFv antibody was produced before the oxygen was displaced and significant amounts thereof were produced after the displacement.

**Example 2: Recombination-mediated post-harvest production
 in transgenic potatoes**

The cDNA described in Artsaenko et al. (1998) which codes for an scFv antibody localized in the endoplasmic reticulum, was modified by means of a linker ligation as described in Example 1, such that it had an NcoI restriction site at the 5' end and an XbaI restriction site at the 3' end. This nucleic acid sequence was inserted in the plasmid pRT100 in the polylinker sequence. The plasmid pRT100/scFv(ox) was obtained. A synthetic nucleic acid was inserted in the NcoI restriction site of pRT100/scFv(ox) which contains two FRT recombination sites (Buchholz et al., Nucleic Acids Res. 24 (1996), 3118-3119). The plasmid pRT 100/FRT-scFv(ox) was obtained. The cDNA coding for the FLP-recombinase LBD fusion protein (WO 95/00555) was inserted as PCR-adapted NcoI-XbaI fragment in the NcoI cleavage site of pRT 100 in the sense orientation behind the CaMV 35S promoter (Odell et al., Nature 313 (1985), 810-812) (plasmid pRT 100/FLP). The following primer pair was used for this purpose:

NcoI-PLP-LBD primer: CATGCCATGCCACAATTTGATATATTATGTAAAC
FLP-LBD-XbaI primer: GCTCTAGATCAGACTGTGGCAGGGAAACCTC

The expression cassette from pRT 100/FLP was inserted as partially digested PstI fragment between the two FRT recombination sites. As a result, the plasmid pRT 100/rec-scFv(ox) was obtained.

Following cleavage with HindIII the expression cassette from pRT 100/rec-scFv(ox) was isolated and inserted in the binary vector pSR 8-30 (Düring et al., Plant Journal 3 (1993), 587-598; Porsch et al., Plant Molecular Biology 37 (1998), 581-585). The expression vector pSR 8-30/rec-scFv(ox) was obtained. Transgenic potato plants were produced as described in Example 1.

For detecting the expression of the scFv antibody, *in vitro* cultivated potato plants were reacted on a medium with 10^{-6} M estradiol (Sigma Chemicals, St. Louis, MO, U.S.A.) and cultivated for 2 days. Thereafter, the plant material was harvested and mortared. The detection of expressed scFv antibodies was again made via the contained c-myc Tag (cf. *supra*) or protein L in Western blot or ELISA. For this purpose, the whole protein of the potato material was isolated and used in the corresponding detection methods.

Potato plants of lines determined to be expression positive were removed from the clonal *in vitro* culture and planted out in a greenhouse. These individual plants had not yet been treated with estradiol so that no recombination had taken place yet. Likewise the cultivation in the field was made under common agricultural conditions. The tubers were harvested as usual and stored. For the post-harvest production of the scFv antibody the tubers were incubated with an atomized solution of 10^{-6} M estradiol in a reaction container. The technical realization was as described in above Example 1. After 40 hours, the tubers were removed from the reaction container, homogenized, the solids content was centrifuged off and the aqueous supernatant was supplied to the chromatographic purification of the scFv antibody. By comparing samples before and after they were contacted with estradiol it could be shown that no scFv antibody was

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8   PRODUCTION OF PROTEINS IN HOST ORGANISMS
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VERIFICATION SUMMARY

DATE: 05/22/2002

PATENT APPLICATION: US/09/889,686B

TIME: 13:45:37

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L:14 M:271 C: Current Filing Date differs, Replaced Current Filing Date

FILET-989686B

09/889686

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BULOW, Lorenz

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PRODUCTION OF PROTEINS IN HOST ORGANISMS

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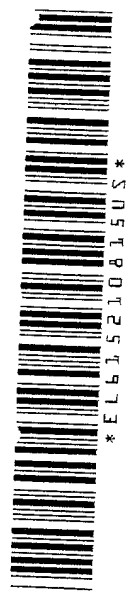
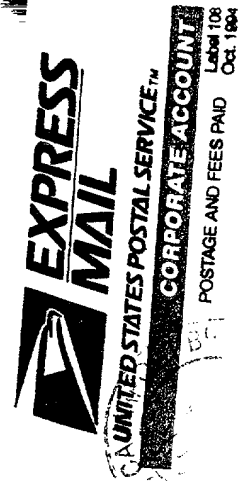
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UNITED STATES POSTAL SERVICE

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POST OFFICE
TO ADDRESSEE



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TO FILE A CLAIM FOR DAMAGE OR LOSS OF CONTENTS, YOU MUST PRESENT THE ARTICLE, CONTAINER, AND PACKAGING TO THE USPS FOR INSPECTION.				X			
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Rec'd PCT/PTO 28 SEP 2001
09/889686

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:

Düring, *et. al*

Application Serial No. 09/889,686

Filed: July 17, 2001

For: **METHODS FOR THE CONTROLLED POST-
HARVEST PRODUCTION OF PROTEINS IN
HOST ORGANISMS**

Attorney's Docket No:
035280133PCUS00

STATEMENT REGARDING SEQUENCE SUBMISSION

BOX/DO/EO/US
Commissioner for Patents
Washington, D.C. 20231

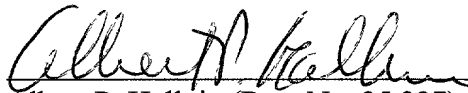
Sir:

In accordance with 37 C.F.R. § 1.821(f), the paper copy of the Sequence Listing and the computer readable copy of the Sequence Listing submitted herewith in the above-mentioned application are the same.

In accordance with 37 C.F.R. § 1.821(g), this submission includes no new matter.

Respectfully submitted,

Date: November 28, 2001


Albert P. Halluin (Reg. No. 25,227)
Viola T. Kung (Reg No. 41,131)

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09/889686

SEQUENCE LISTING

<110> DÜRING, Klaus
BÜLOW, Lorenz

<120> METHOD FOR THE CONTROLLED POST-HARVEST
PRODUCTION OF PROTEINS IN HOST ORGANISMS

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Claims

1. A method of obtaining a desired protein from a transgenic host organism, wherein the expression of the gene coding for this protein is not made until the host organism has been harvested, characterized in that
 - (a) the transgenic host organism contains the gene coding for the desired protein such that it is only expressed in the presence of a chemical inductor; and
 - (b) contacting with the inductor takes place via the phase surrounding the host organism after the host organism has been harvested.
2. The method according to claim 1, wherein the phase is a gas phase.
3. The method according to claim 1, wherein the phase is a liquid phase.
4. The method according to claim 2, wherein step (b) comprises modification of the gas phase surrounding the host organism, atomization of a solution (suspension) of an inductor or flooding with a volatile inductor.
5. The method according to claim 3, wherein step (b) comprises an infection with a virus suspension.
6. The method according to any of claims 1 to 5, wherein the gene coding for the desired protein is functionally linked with an inducible promoter.
7. The method according to any of claims 2, 4 and 6, wherein the modification of the gas phase is an deoxidation and the promoter is a promoter inactive under aerobic conditions.
8. The method according to claim 7, wherein the promoter is the GapC4 promoter.

0999666-112001

9. The method according to any of claims 2, 4 and 6, wherein in step (b) contacting with the chemical inductor takes place via atomization of the inductor RH5992.
10. The method according to any of claims 1 to 3, wherein the expression of the gene coding for the desired protein is induced by compensating the functional inhibition of the transcription and/or translation.
11. A method according to claim 10, wherein the gene coding for the desired protein is functionally linked with a promoter, so that between the promoter and the gene a nucleic acid is inserted which is characterized in that
 - (a) it prevents the transcription and/or translation of the gene; and
 - (b) it can be excised after the induction, which results in the expression of the gene.
12. The method according to claim 11, wherein the nucleic acid is a nucleic acid which can be excised by an inducible recombinase.
13. The method according to claim 12, wherein the excisable nucleic acid and the recombinase are constituents of the recombinase-LBD system.
14. The method according to any of claims 1 to 3, wherein the gene coding for the desired protein is expressed by compensating the effect of the transcriptional, post-transcriptional, translational or post-translation repressor.
15. The method according to any of claims 1 to 14, wherein the method is a combination of at least two of the methods defined in claims 6, 10, 11 and 14.
16. The method according to any of claims 1 to 15, wherein the transgenic organism is a useful plant.

17. The method according to claim 16, wherein the useful plant is wheat, barley, corn, sugar beet, sugarcane, potato, a brassicaceae, a leguminous plant or tobacco.
18. A host organism according to claim 1, which contains the gene coding for the desired protein such that it is only expressed in the presence of a chemical inductor.

09999999 112801

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1000

Combined Declaration and Power of Attorney for Patent Application

Docket Number: 03528.0133.PCUS00

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter that is claimed and for which a patent is sought on the invention entitled Method for the Controlled Post-Harvest Production of Proteins in Host Organisms, the specification of which is attached hereto unless the following box is checked:

☒ was filed on July 17, 2001;
as United States Application Number or PCT International Application Number 09/889,686; and
was amended on July 17, 2001 (if applicable).

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information that is material to patentability as defined in 37 C.F.R. § 1.56.

I hereby claim foreign priority benefits under 35 U.S.C. § 119(a)-(d) or § 365(b) of any foreign application(s) for patent or inventor's certificate, or § 365(a) of any PCT international application, which designated at least one country other than the United States listed below, and have also identified below any foreign application for patent or inventor's certificate, or PCT international application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)

Priority Claimed

PCT/DE00/03119
(Application No.)

WO
(Country)

September 2000
(Day/Month/Year Filed)

☒ Yes ☐ No

199 56 272.5
(Application No.)

DE
(Country)

23 November 1999
(Day/Month/Year Filed)

☒ Yes ☐ No

I hereby claim the benefit under 35 U.S.C. § 119(e) of any United States provisional application(s) listed below.

(Application No.)

(Filing Date)

(Application No.)

(Filing Date)

I hereby claim the benefit under 35 U.S.C. § 120 of any United States application(s), or under § 365(c) of any PCT international application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT international application in the manner provided by the first paragraph of 35 U.S.C. § 112, I acknowledge the duty to disclose information that is material to patentability as defined in 37 C.F.R. § 1.56 that became available between the filing date of the prior application and the national or PCT international filing date of this application.

(Application No.)	(Filing Date)	(Status - patented, pending, abandoned)
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(Application No.)	(Filing Date)	(Status - patented, pending, abandoned)
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I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

Jason C. Abair, Reg. No. 44,007
Ethan B. Andelman, Reg. No. 48,997
Michael J. Bell, Reg. No. 39,604
Luisa Bigornia, Reg. No. 45,974
Andrew S. Brenc, Reg. No. 45,534
Celine T. Callahan, Reg. No. 34,301
Jenny W. Chen, Reg. No. 44,604
Robin C. Chiang, Reg. No. 46,619
Chris Comuntzis, Reg. No. 31,097
Thomas E. Coverstone, Reg. No. 36,492
James F. Davis, Reg. No. 21,072
Thomas M. Dunham, Reg. No. 39,965
Panpan Gao, Reg. No. 43,626
Darren J. Gold, Reg. No. 47,599
Alan M. Grimaldi, Reg. No. 26,599
Jay Guiliano, Reg. No. 41,810
Albert P. Halluin, Reg. No. 25,227
Leslie L. Jacobs, Jr., Reg. No. 40,659
Derek J. Jardieu, Reg. No. 44,483

Christopher L. Kelley, Reg. No. 42,714
Brian S.Y. Kim, Reg. No. 41,114
Viola T. Kung, Reg. No. 41,131
Robert C. Laurenson, Reg. No. 34,206
Joseph P. Lavelle, Reg. No. 31,036
Don F. Livornese, Reg. No. 32,040
Christopher A. Mathews, Reg. No. 35,944
Matthew J. Moore, Reg. No. 42,012
Andrew Y. Piatnicia, Reg. No. 40,772
William C. Rooklidge, Reg. No. 31,791
Michael J. Stimson, Reg. No. 45,429
William K. West, Reg. No. 22,057
Justin A. White, Reg. No. P-48,883
Adam K. Whiting, Reg. No. 44,400
Jayna R. Whitt, Reg. No. 47,175
Karen K. Wong, Reg. No. 44,409
Wallace Wu, Reg. No. 45,380
Matthew S. Zises, Reg. No. 47,246

37

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Facsimile: (650) 463-8400

Direct Telephone Calls to: (650) 463-8109

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Full name of sole or first inventor Klaus DÜRING	
Inventor's signature <i>Düring</i>	Date <i>14.11.2011</i>
Residence Vorgebirgsweg 33, D-50226 Frechen-Königsdorf, GERMANY <i>DEX</i>	
Citizenship DE	
Mailing Address Vorgebirgsweg 33, D-50226 Frechen-Königsdorf, GERMANY	
Full name of second inventor Lorenz BÜLOW	
Second inventor's signature <i>Bülow</i>	Date <i>14.11.2011</i>
Residence Werder 2, D-38100 Branschweig, GERMANY <i>DEX</i>	
Citizenship DE	
Mailing Address Werder 2, D-38100 Branschweig, GERMANY	

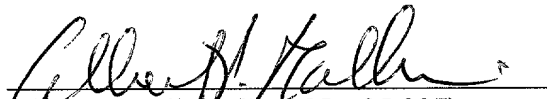
(Supply similar information and signature for subsequent joint inventors, if any)

09/889686

such extensions of time are hereby petitioned, and any fees therefor are hereby authorized to be charged to our Deposit Account No. 08-3038 referencing docket number 03528.0133.PCUS00.

Respectfully submitted,

Date: April 10, 2002


Albert P. Hallum (Reg. No. 25,227)
Viola T. Kung (Reg. No. 41,131)

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Box No. 34
301 Ravenswood Avenue
Menlo Park, CA 94025
Tel. (650) 463-8109
Fax (650) 463-8400

Adjustment date: 04/18/2002 UEDUWJE
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03 FC:969 -135.00 DP

04/18/2002 UEDUWJE 00000140 09889686
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09/889,686

PCT/DE00/03119

03528.0133.PCUS00

24. The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :

- ☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO \$1040.00
- ☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO \$890.00
- ☐ International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO \$740.00
- ☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1)-(4) \$710.00
- ☐ International preliminary examination fee (37 CFR 1.482) paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) \$100.00

ENTER APPROPRIATE BASIC FEE AMOUNT =

\$0.00

Surcharge of \$130.00 for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).

\$130.00

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE
Total claims	32 - 20 =	12	x \$18.00
Independent claims	1 - 3 =	0	x \$80.00
Multiple Dependent Claims (check if applicable).			<input type="checkbox"/>

TOTAL OF ABOVE CALCULATIONS =

\$346.00

☒ Applicant claims small entity status. See 37 CFR 1.27). The fees indicated above are reduced by 1/2.

\$173.00

SUBTOTAL =

\$173.00

Processing fee of \$130.00 for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)).

\$0.00

TOTAL NATIONAL FEE =

\$173.00

Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (check if applicable).

☐

\$0.00

TOTAL FEES ENCLOSED =

\$173.00

Amount to be:

refunded

\$

charged

\$

- a. ☒ A check in the amount of \$173.00 to cover the above fees is enclosed.
- b. ☐ Please charge my Deposit Account No. _____ in the amount of _____ to cover the above fees. A duplicate copy of this sheet is enclosed.
- c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. 08-3038 A duplicate copy of this sheet is enclosed.
- d. ☐ Fees are to be charged to a credit card. **WARNING:** Information on this form may become public. **Credit card information should not be included on this form.** Provide credit card information and authorization on PTO-2038.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

12/06/2001 HNGUYEN 00000051 09889686

01 FC:254

65.00 OP

02 FC:967

108.00 OP

Albert P. Halluin

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Box 34

Menlo Park, CA 94025

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SIGNATURE

Albert P. Halluin, Viola T. Kung

NAME

25,227, 41,131

REGISTRATION NUMBER

November 28, 2001

DATE